Salt Tolerant Tissue of Taro: Selection and Constituents

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ABSTRACT

A salt-tolerant tissue was selected from callus of Colocasia esculenta var. antiquorum initiated on solid Knop's medium by subculturing for 4 years on modified Linsmaier-Skoog medium containing 100-700 milliosmoles (10%-70%) artificial seawater. Plantlets were initiated by transferring the tissue to TIBA-containing solid medium.

Cell wall thickness, cell size, chloroplast size and structure, and starch grains are affected by the concentration of seawater. The same is true for levels of inorganics, calcium oxalate, chlorophyll, protein, and secondary and quarternary alkaloids. These changes may be related to salinity tolerance and adaptation.

Introduction

A number of workers have successfully applied contemporary tissue culture techniques to taro (Hartman, 1974; Mapes and Cable, 1972; for a review see Arditti and Strauss, 1979). However, response to in vitro conditions is genotype specific (Jackson, Ball and Arditti, 1977). Of those cultivars reported to produce callus from shoot tip explants, most have low percentages of regeneration and the callus is not easily maintained (Jackson, Ball and Arditti, 1977; Nyman, Gonzales and Arditti, 1981).

Callus of a cultivar of Colocasia esculenta var. antiquorum name UCI Runner is one of the least recalcitrant genotypes. Shoot tip explants from this cultivar produce a callus which undergoes a rapid, but reversible transition to a stable differentiated growth form we have termed "calloid" (Nyman, Gonzales and Arditti, 1981; Nyman, Gonzales and Arditti, in press a) and b). The transition from callus to calloid appears to represent a prerequisite physiological event necessary for organogenesis. Unlike most taro calli, calloid masses are stable, fast growing and organogenetically active. These characteristics allow for the selection of salt tolerant tissues and plantlets (Nyman, Gonzales, and Arditti, 1981).

The purpose of this study was to: (1) assess the effects of growth regulating substances and media components on organogenesis from calloid tissues, and (2) determine the effects of seawater on differentiation and organogenesis from calloid. Since there is some evidence to suggest that the calloid stage may occur prior to shoot formation from rice (Inoue and Maeda, 1980; Maeda, 1980a and b) and

sugarcane callus (Krishnamurthi, 1977), this information may be useful in developing more direct methods for plantlet production from calli of other monocots.

Materials and Methods

Tissue culture

Callus cultures were produced by previously described methods from shoot tip explants of a cultivar of Colocasia esculenta var antiquorum named UCI Runner (Arditti and Strauss, 1979; Jackson, Ball and Arditti, 1977). Once initiated, callus was transferred to solid or liquid Linsmaier-Skoog medium containing 0.1 mg/l napthaleneacetic acid (NAA) and 0.1 mg/l adenine-N-benzyl-9-tetrahydro-2H-pyran-2-yl (SD8339-Shell Development Co., Modesto CA., 95252, USA) or 1.0 mg/l dimethylaminopurine (6-DMAP). Tissues were subcultured on the maintenance medium every 2 to 4 months under conditions described earlier (Nyman, Gonzales, and Arditti, in press a).

Salt tolerance was selected for by transferring tissues to successively higher concentrations of an artificial seawater mix added to the culture medium (Nyman, Gonzales, and Arditti, in press b; Nyman, Gonzales and Arditti, 1981). The components and methods for preparing the artificial seawater (ASW) are available in the literature (Cavanaugh, 1964; Nyman, Gonzales, and Arditti, 1981). To obtain plantlets, tissues were transferred to media containing substitutions of the the hormones present in the LS maintenance medium and the cultures were observed daily. The growth regulating substances used in the experiments included: kinetin (K), benzylaminopurine (BAP), adenine-N-benzyl-9-tetrahydro-2H-pyran-2-yl (SD8339), 6-dymethylaminopurine (6-DMAP), napthaleneacetic acid (NAA), indoleacetic acid (IAA), 2, 4, 5-trichloroacetic acid (2, 4, 5-T), gibberellic acid (GAA), 2, 4, 5-tri-iodobenzoic acid (TIBA) and aminocyclopropane-1-carboxylic acid (ACCP). Some of these compounds were also tested on whole plantlets in an attempt to enhance shoot and/or root development (Table 2). As well, dilution of components in the LS medium and alternate media were tested for their effects on plantlet development (Tables 1 and 2).

In attempts to further enhance plantlet development, equimolar substitutions of fructuose, raffinose, galactose, and sorbitol were made for sucrose in the LS medium. Anticontaminants singly and in combinations were also tested for their effects on calloid differentiation. These included benlate, Nystatin, Penicillin G, Gentamycin, sodium omadine, Kanamycin sulfate, Vancomycin, and Amphotericin B.

Microscopy

Plant material for scanning electron microscopy was cut into 1 mm^3 pieces and fixed for 14 to 18 hours in 4% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.2. After a slow ethanol dehydration (10% increments for 20 to 30 minutes at each concentration), and a similarly slow infiltration with freon, the tissue was dried in a Bomar 900 EX critical point dryer. The material was then mounted and coated with gold for 3 minutes in a Hummer 2 Sputter coater. Observations were made with an Hitachi S-500 scanning electron microscope at 5 KV.

To stabilize tissues taken from samples grown in seawater media, sucrose was added to the fixation solution. A concentration of 0.63M sucrose was empirically found suitable for tissues grown on 70% ASW. Concentrations of sucrose necessary to stabilize tissues from other levels of ASW were calculated mathematically (i.e. 0.09M sucrose/10% ASW, 0.18M sucrose/20% ASW).

Results

Plantlet differentiation from calloid

After 32 weeks, plantlets differentiated from calloid grown on group IIa maintenance media (Figure 1, Table 1). However, most of these had developed root primordia visible only with the use of light and scanning electron microscopes (Figure 2). The first formed leaves were linear in appearance (Figure 2) and plantlets did not grow vigorously. As well as generating plantlets, calloid tissues produced various types of abnormal leaf and shoot-like structures, and protocorm-like bodies (Figures 3 and 4).

Calloid could not be maintained on media lacking either an auxin or a cytokinin or both. High levels of auxin were damaging to the calloid leading to necrosis and death (Table 1). Although GAA added to IIa media at low concentrations enhanced growth, it did not affect either the rate or amount of plantlet differentiation.

Tri-iodobenzoic acid (TIBA) added at 1 mg/l to media without hormones caused a stimulation of plantlet production and a corresponding decrease in the numbers of abnormal structures (Figure 5, Table 1). Further, plantlets were produced in a much shorter period (2 to 4 weeks) and grew more vigorously than those on other media (Table 1). In the presence of K, TIBA did not stimulate normal plantlet production, but calloid grew as a light green mass of tiny compressed shoots (Figure 6).

Development of individual plantlets

Plantlets produced from calloid cultures appeared variable in: (1) appearance of their shoot systems, (2) degree of root formation, and (3) rate of growth. However, some characteristics of their development were uniformly affected by changes in media composition (Table 2). Growth of individual plantlets was supported by half strength and full strength media without exogenous hormones, including LS, Murashige-Skoog (MS) and soil extract (SE). Full strength media favored development of plantlets with relatively large spatulate leaves and thick petioles (Figure 7). Shoots were less well developed on half strength media, but roots were formed at a faster rate (Figure 8).

In presence of K and/or NAA, shoots developed more slowly than on media without hormones, producing higher numbers of linear leaves (Table 2). The addition of the auxin 2, 4, 5-T caused either severe retardation of shoot growth or plantlet death. Plantlets grown in the presence of the ethylene precursor, ACCP at concentrations between 0.1 to 1.0 mg/l were not visibly different from those on hormone-free medium. At a concentration of 10 mg/l, ACCP was lethal to plantlets. The addition of 1 mg/l TIBA both with and without K did not appear to affect individual plantlet development (Table 2).

Effects of artificial seawater on calloid and plantlets

Calloids were established and grew well in concentrations of ASW from 100 to 700 milliosmoles (10% to 70%). Although no differences in gross morphology were noted in calloid tissues, optimum plantlet production occurred at 40% to 50% ASW on group IIa media (Table 1). Plantlets differentiated after approximately 32 weeks of growth for all seawater concentrations, and were produced on both solid and liquid media. Development was more rapid on solid medium.

Table 1. Maintenance and media for differentiation of plantlets from calloid cultures.

Use	Basal medium @	State of medium	Growth regulators (mg/l) cytokinin#	auxin*	gibberellin**	other***	Results
Maintenance of calloid (Group I)	LS	Solid	0	0	0	0	Necrosis and death often occurred within the first month of culture
Maintenance of calloid (Group IIa)	LS	Solid	1.0 BAP	0.1 NAA	0	0	Calloid healthy and grew well--plant let differentiation after 32 weeks, few roots, shoots superficially like seedlings, but first formed leaves linear and plantlets very small--some abnormal growth
	LS	Solid	1.0 SD8339	0.1 NAA	0	0	
	LS	Solid	1.0 6-DMAP	0.1 NAA	0	0	
	LS	Solid	1.0 K	0.1 NAA	0	0	
	LS	Solid	1.0 SD8339	0.1 NAA	0.1 GAA	0	Calloid appeared dark green and grew well, but plantlet differentiation was similar to media IIa without GAA
	LS	Solid	1.0 SD8339	0.5 NAA	0.1 GAA	0	
	LS	Solid	0.2 BAP	0	0	0	Necrosis and death
	LS	Solid	0	0.2 NAA	0	0	Necrosis and death
Maintenance of calloid (Group IIb)	LS	Solid and liquid	10.0 BAP	0 NAA	0	0	Chlorosis and poor growth rate
	LS	Solid and liquid	10.0 BAP	2.0 IAA	0	0	Chlorosis and poor growth rate
	LS	Solid and liquid	10.0 BAP	0	0	0.1 ACCP	Chlorosis and poor growth rate--some death
	LS	Solid and liquid	10.0 BAP	0	0	1.0 ACCP	Chlorosis and poor growth rate--some death
Maintenance of calloid and stimulation of vigorous plantlet growth (Group III)	LS	Solid	0	0	0	1.0 TIBA	Stimulation of plantlet development after 2 to 4 weeks in culture--plantlets larger with more normal looking spatulate leaves more numerous plantlets with visible roots
	LS	Solid	0	0	0	50 to 100 TIBA	Necrosis and death
	LS	Solid	1.0 K	0	0	1.0 TIBA	Light-green color --many small compressed plantlets with linear leaves, rapid calloid growth

@ For composition of media, see Arditti and Strauss, 1979; LS-Linsmaier-Skoog medium
# BAP-benzylaminopurine, SD8339- adenine-N-benzyl-9-tetrahydro-2H-pyran-2-yl 6-DMAP-6-dimethylaminopurine
*IAA- indoleacetic, NAA- napthaleneacetic acid
**GAA- gibberellic acid
***ACCP- aminocyclopropane-1-carboxylic acid, TIBA- 2, 4, 5-tri-iodobenzoic acid

Note: Other concentrations of cytokinins and auxins used in media IIa, but since these do not significantly affect growth rate of calloid or plantlets, they are not listed separately. Concentrations of 0.1 mg/l have been tested for all cytokinins with 0.1 mg/l NAA.

Table 2. Maintenance and media for plantlets derived from calloid cultures

Use	Basal medium @	State of medium	Growth regulators (mg/l) cytokinin#	auxin*	gibberellin**	other***	Results
Maintenance of small plantlets without added hormones (Group I)	½ strength LS, MS, and SE	Solid	0	0	0	0	Most plantlets developed shoot systems superficially resembling seedling shoots, leaves were spatulate in appearance some developed visible roots after 1 to 3 months
	full strength LS, MS, and SE (with and without Nuchar)	Solid	0	0	0	0	Full strength media favoured shoot development, ½ strength root development
Maintenance of small plantlets and root stimulation (Group II)	full strength LS, MS and SE	Solid	0.6 K	6.0 NAA	0	0	Vegetative growth of shoots is slightly reduced over group I- leaves appeared more linear in shape, slight promotion of root growth
			1.0 K	2.0 NAA	0	0	
			3.0 K	2.0 NAA	0	0	Other concentrations yield similar results as above
	full strength LS	Solid & liquid	1.0 K	1.0 NAA	0	0.1 ACCP	Reduced shoot growth--compared to group I leaves linear
			0	1.0 NAA	0	0.1 ACCP	Plantlets similar to group I
			0	0	0	1.0 ACCP	Plantlets similar to group I
			0	0	0	10.0 ACCP	Necrosis and rapid plantlet death
			0	0.2 2,4, 5-T	0	0	Reduced growth rate of shoots and roots- some necrosis and death
Maintenance of small plantlets (Group III)	½ strength LS	Solid	0	0	0	1.0 TIBA	Similar growth to group 1
	½ strength LS	Solid	1.0 K	0	0	1.0 TIBA	Similar growth to group 1
	½ strength LS with nuchar	Solid	1.0 K	0	0	1.0 TIBA	Similar growth to group 1
	½ strength LS with nuchar	Solid	0	0	0	1.0 TIBA	Similar growth to group 1

@ For composition of media, see Arditti and Strauss, 1979; LS- Linsmaier-Skoog medium; MS- Murashige-Skoog medium; SE- soil extract medium.

K-kinetin

* NAA- napthaleneacetic acid, 2, 4, 5-T - 2, 4, 5- trichloroacetic acid

**GAA- gibberellic acid

***ACCP- aminocyclopropane-1-carboxylic acid, TIBA- 2, 4, 5-tri-iodobenzoic acid

Plantlet production was stimulated on LS medium by the addition of 1 mg/l TIBA (Table 1). Rate of plantlet production was much improved on this medium, and differentiation of the first visible plantlets occurred after 2 to 6 weeks. Although this enhancement of plantlet production was noted on all seawater concentrations, plantlets produced on 60% and 70% ASW were often smaller and grew less vigorously than those on 50% and below.

Observations of 32-week-old calloids made under the SEM show protocorm-like bodies (Figure 4), abnormal shoot and leaf-like structures (Figure 3), as well as normal-looking plantlets were produced on all seawater concentrations. Most calloid masses had at least some differentiated stomata on regions other than leaf primordia (Figure 9). However, development of large calloid surfaces with high numbers of stomata was more common on tissue grown in liquid rather than on solid cultures. Numbers of stomata were highest on calloids grown in 40% ASW liquid cultures.

Regions with a tumor-like appearance were found on calloid masses at all ASW concentrations (Figure 10). Variations in the structure of these occurred both within and between cultures. However, all appeared to result from two or more growth centers existing in close proximity.

Discussion

Some calloids grown on standard LS maintenance medium with 1.0 mg/l DMAP and 0.1 mg/l NAA develop plantlets after 4 to 6 months (Nyman and Arditti, in press b). More commonly, however, 32 weeks were required before calloid cultures produced significant numbers of normal-looking plantlets under these conditions.

In the present study, observations of calloid from LS maintenance medium under the SEM, revealed abnormal leaf-like and shoot-like growth reminiscent of structural abnormalities associated with lateral buds exposed to artificially high auxin/cytokinin ratios (Nyman and Cutter, 1981). Among such effects are development of shoots with decreased or missing leaf primordia, and reduction in mature leaf size. Occurrence of linear leaf-like structures arising from calloid tissues and plantlets and the observed protocorm-like bodies may be growth forms representative of an imbalance in the auxin/cytokinin levels. The tumor-like structures may be caused by a number of meristems arising in close proximity which become stunted and do not produce normal sequences of leaf primordia.

Calloid characteristics indicative of supraoptimal auxin concentrations could not have been observed without high resolution microscopy studies. These observations led to experimental use of four different media containing TIBA, a known antiauxin. The results with low concentrations of TIBA were consistent with theoretical considerations in that normal plant development indicative of optimal auxin concentration was favored over abnormal growth. There results indicate that structural observations can be used in the formulation of new media.

Root development was favored by half strength media, whereas full strength media were better suited for shoot development. Good root development on a two-fold dilution of basal medium has also been reported for Douglas fir plantlets regenerated from cotyledons (Cheng, 1979). Growth effects of media components may vary depending on a number of factors (Dougall, 1981). Thus, it is possible that dilution effects may be due to reduced levels of one or more components or general osmotic effects.

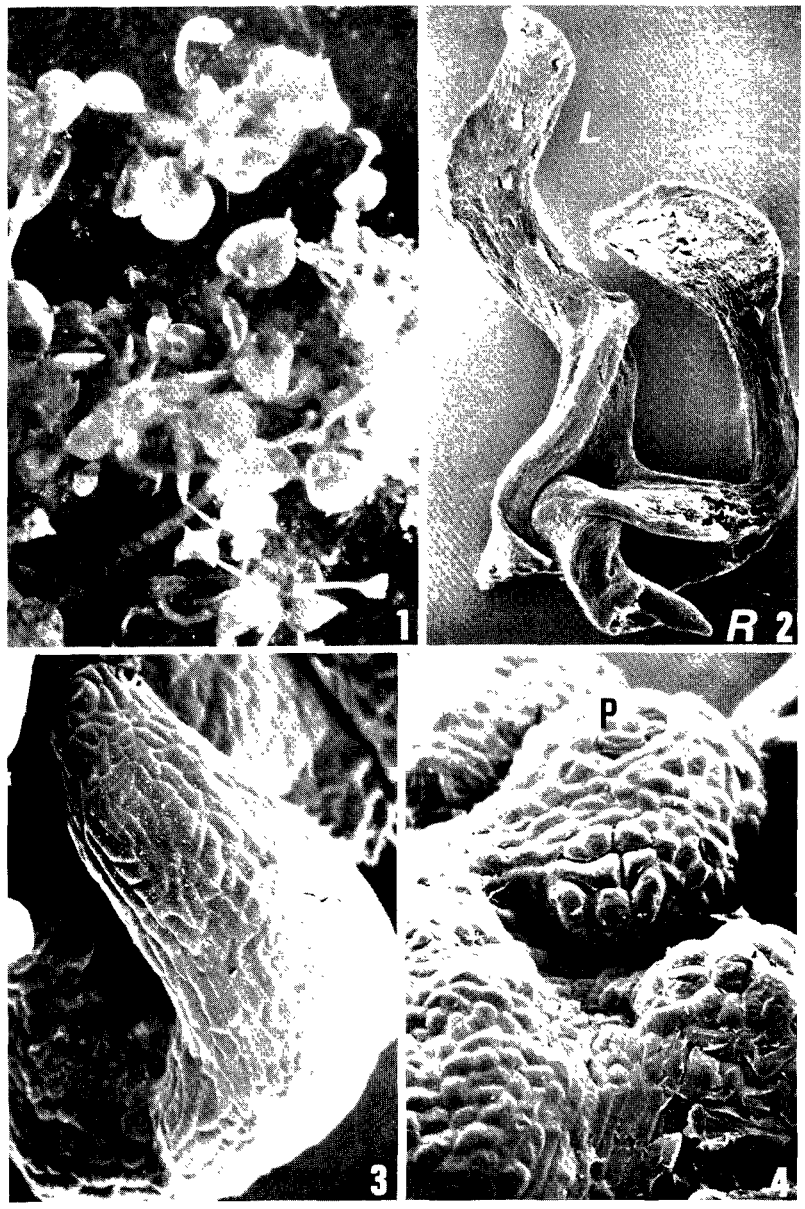

Figure 1. Plantlets differentiating from calloid after 32 weeks in culture and placed on Linsmaier-Skoog medium without added hormones. The calloid from which the plantlets were produced had been maintained on Linsmaier-Skoog medium with 1 mg/l 6-dimethylaminopurine and 0.1 mg/l napthaleneacetic acid. x 5.

Figure 2. Scanning electron micrograph of a single plantlet produced from calloid grown on Linsmaier-Skoog medium with 1 mg/l 6-dimethylaminopurine and 0.1 mg/l napthaleneacetic acid. Note the linear appearance of the first formed leaves (L) and the small root primordia (R). x 19.

Figure 3. Scanning electron micrograph of a leaf-like structure produced by a 32 week old callodi growing on the same medium as Figure 2. x 160.

Figure 4. Scanning electron micrograph of protocorm-like (P) in a young calloid ($\sim$ 12 weeks old) growing on the same medium as Figure 2. x 200.

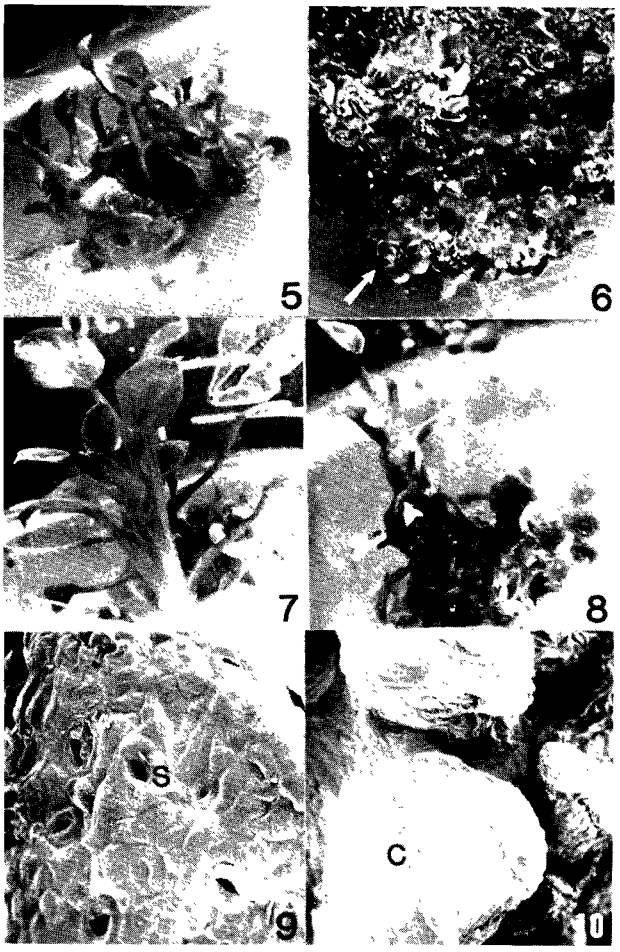

Figure 5. Differentiating plantlets from a calloid culture growing for 4 weeks on Linsmaier-Skoog medium in the presence of 1 mg/l tri-iodobenzoic acid. x 4.

Figure 6. Calloid growing for 4 weeks on Linsmaier-Skoog medium in the presence of 1 mg/l tri-iodobenzoic acid and 1 mg/l kinetin. Note the numerous tiny compressed plantlets (arrow). x 4.

Figure 7. Individual plantlet growing on full strength Linsmaier-Skoog medium for 2 weeks, transferred from a culture similar to the one shown in Figure 5. x 4.

Figure 8. Individual plantlet growing in half strength Linsmaier-Skoog medium for 2 weeks, transferred from a culture similar to the one shown in Figure 5. x 4.

Figure 9. Scanning electron micrograph of the surface of a calloid from Linsmaier-Skoog medium with 1.0 mg/l dimethylamilnopurine and 0.1 mg/l napthaleneacetic acid, and 100 milliosmoles (10%) artificial seawater. Note the abundant stomata (S). x 400.

Figure 10. Tumor-like region on a calloid surface from Linsmaier-Skoog medium with 1.0 mg/l dimethylaminopurine and 0.1 mg/l napthaleneacetic acid, and 300 milliosmoles (30%) artificial seawater. Note the closely spaced growth centers (C).x 60.

The concentrations and chemical nature of auxins and cytokinins as well as the addition of ACCP to culture media affected plantlets and calloid differently. NAA was more effective than IAA in media used to support the calloid, possibly due to its stability. Calloid growth in the present of the four cytokinins tested was equivalent suggesting that all are equally effective. In the absence of either an auxin or a cytokinin calloids became necrotic and died. This indicates that requirements of calloid are for a balanced auxin-cytokinin ratio. The effect of TIBA may be to support a favorable auxin/cytokinin balance in the tissue. This view is supported by observations that normal plantlet development is inhibited when TIBA IS added in the presence of exogenous kinetin. Further if TIBA affects auxin levels, then it is reasonable to assume that endogenous auxin/cytokinin levels are not optimal. All hormone effects observed were clearly independent of ASW concentration.

At this point, it is difficult to determine whether the adaptation to ASW is genotypic or phenotypic. However a number of general characteristics should be noted: (1) cumulative growth on all concentrations (except for 60% ASW) was comparable; (2) in the initial cultures, higher numbers of plantlets with a normal appearance formed on 40% ASW; (3) calcium oxalate content, chlorophyll, protein and secondary alkaloid levels in ASW cultures were higher than those on standard medium (Nyman, Gonzales, and Arditti, 1981), and (4) cell wall thickness, cell size, chloroplast size and structure, numbers and size of starch grains appear to vary with ASW concentration (unpublished results). A great deal of variation was also noted in plantlet characteristics. This variation was independent of medium and therefore appears to be somaclonal (Larkin and Scowcroft, 1981). If so, the basis of adaptation to ASW cultures may be genetic and the strategy for production of salt tolerant cultivars might include the screening of somaclonal variants.

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